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13. ABSTRACT *(Maximum 200 words)*

Several achievements are made through this initial collaborative project. These are: 1) creation of enormous capability at this HBCU in enhancing this research; 2) extensive studies on identification of TNT biotransformation products in several bacterial species and one fungal species; 3) identification of several genes which are capable of catalyzing degradation of metabolites of TNT in several microbial species, particularly in the fungus *P. chrysosporium*; 4) isolation and DNA sequence of these genes for probe development; and 5) PCR amplification of 2,4-DNT gene using *P. chrysosporium* genomic DNA as template; 6) DNA:DNA hybridization ^{32}P labeled 2,4-DNT dioxygenase gene (used as probe) to anneal with *P. chrysosporium* genomic DNAs; and 7) selection of proper combination of mixed genotypes of bacteria for very efficient degradation of TNT.

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I. INTRODUCTION

Bioremediation has been proposed as a strategy to clean TNT contaminated sites. Some bacterial species, particularly *Pseudomonas* are capable of growing in TNT concentration that are fatal to fungi . For example, if the TNT concentration is greater than 50 ppm the fungus *Phanerochaete chrysosporium* is killed, however this fungus can mineralize TNT in pure culture though little is known about the pathway and genes involved in the fungus.

The primary goal of this project is to create a microbial system capable of mineralizing TNT in sites with high TNT levels. Apparently a cluster of genes are involved in TNT degradation. Only a few of these genes have been characterized. TNT is non-specifically transformed to isomers of mono-aminodinitrotoluene (Am-DNT). The genes involved in DNT (Suen and Spain, 1993) and toluene mineralizations are well characterized (Fanklin et al., 1981) but the genes which degrade Am-DNT are unknown.

II. NATURE OF COLLABORATION

This initial project was the result of a Broad Agency Announcement from Army with three primary goals: 1) Build up a collaboration with the ongoing activities of an Army Laboratory. This was possible due to creation of collaboration (mostly as advisor) with Dr. Douglas Gunnison, Sr. Microbiologist of Army Corps of Engineers at WES. 2) Provide help to a historically black college or university (HBCU) in building up the capability in bioremediation research. 3) Identify genes which code for TNT degradation in bacterial and fungal organisms.

III. OBJECTIVES

The specific technical objectives of this work unit are as follows: (1) Enrichment and isolation of TNT degrading microbial cultures; (2) Characterization of gene(s) involved in the TNT degradation mechanism. These degradation genes will be characterized in both the white rot fungus and bacterial isolates.

IV. TNT RESEARCH CAPABILITIES CREATED AT THIS HBCU

Howard University, as part of it's commitments to this collaboration has invested $60,000 in a new plant and soil research laboratory to work exclusively with TNT and related toxic chemicals. These facilities include: new HPLC (high performance liquid chromatography) system, two plant growth chambers, facilities for anaerobic microbiology and PCR..

Following pre- and post-doctoral students trained in molecular biology techniques, mostly on voluntary basis except a few who received remuneration as identified below:

Undergraduate Students:

Daphne Jones
Nicole R. Holmes
Erik Watson
Benjamin Dyson Jr. (paid from Army project)
Adebisi Ajala
Khaled S. Mirza (paid from Army project)
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Graduate Students:

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Hirendra N. Banerjee (partly paid from Army project)
Gregory Davenport
James Stewart
Anhtuan L. Truong
Gail Hollowell (paid from Army project)

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V. Rangaswamy
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Li-Hua Hou (paid from Army project)
William Gillete
Michael M. LaMontagne
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V. CULTURE COLLECTION AND THEIR ROLES IN TNT DISAPPEARANCE

Bacterial and Fungal Activities for Cell Growth, Peroxidase Activities and Biotransformation:

Bacteria and fungal cultures that grow in presence of TNT are listed in Table 1. In bacterial biomass (Figure 1 and Table 2), protein (Table 3), and NO_2 (Table 4) levels increased in the presence of TNT bacterial cultures. HPLC analysis indicates a non-specific increase in Am-DNT (Figure 2) in all cultures tested. The mechanism responsible for the biotransformation of TNT to Am-DNT has not been elucidated. In fungal culture *Phanerochaete chrysosporium* TNT decreased biomass but the culture reduced TNT concentrations (Table 2).

P. chrysosporium thrives on woody substrate where it effectively degrades lignin. The lignin degrading system involves peroxidase or commonly referred to as lignin peroxidase (LiP) and manganese peroxidase (MnP), as well as H_2O_2-generating peroxidase (Gold et al., 1989; Kirk and Farrell, 1987). Under ligninolytic conditions this fungus can degrade a variety of pollutants such as PCB (polychlorobenzene), 1,1,1-trichloro-2-bis(4-chlorophenyl) ethane (DDT), phenanthrene, 2,4,5-trichlorophenoxyacetic acid, BTEX (benzene, toluene, ethyl benzene, and xylenes) and 2,4,6-TNT (Kohler et al., 1988; Sutherland et al., 1991; Yadav and Reddy, 1992; Spiker et al., 1992). This fungus does not express the extracellular peroxidase under non-ligninolytic conditions. It was essential for us to know the role of peroxidase enzyme in degrading TNT and it's metabolites. We have compared the ability of *P. chrysosporium* to degrade TNT, 2,4-DNT and 2-Am-DNT under ligninolytic and non-ligninolytic conditions. The degradation of 2,4,6-TNT by this fungus has already been reported (Michels and Gottschalk, 1995).

Peroxidase activities in *P. chrysosporium* cells are seen only under lignolytic (low nitrogen) condition (Fernando et al., 1990; Valli et al., 1992). ^{14}C-TNT disappearance (Figure2) and HPLC analysis indicate TNT is degraded under non-ligninolytic condition. These data provide insight, not reported before, with respect 2,4-DNT disappearance, was not dependent on peroxidase enzyme, suggesting that this fungus has genes for 2,4-DNT degradation (Tables 5 and 6).

In this study we have shown that bacterial cultures which were isolated from 2,4,6-trinitrotoluene (TNT) contaminated soil can biotransform TNT by non-specific reduction of NO_2 and form monoaminodinitrotoluene (Am-DNT). This non-specific NO_2 reduction does not happen in killed (inactivated) cells, nor TNT is absorbed by cells. This transformation to Am-DNT is facilitated by the presence of the cometabolite 0.5% NaOAc. When 0.5% NaOAc was omitted, cells in all cultures grew very slowly (OD/600 nm 0.2-0.5). In other laboratories they have used yeast (200 mg/L) extract or glucose. This shows for initial growth, some push from co-metabolities are needed for degradation of TNT and DNT. The ability to degrade Am-DNT appears to be plasmid based. *P. putida* strain 2015 cannot degrade Am-DNT without the presence of the plasmid pWWO (Figure 3).

TABLE 1 . List of cultures used and presence of plasmids in cells.

Names	OD/600 nm				Presence of plasmid	Source
	Control	Killed cells	Days grown 1	Days grown 5		
Pseudomonas aeruginosa	0.00	0.019	1.117	0.086	+	Isolated by Dr. Gunnison
P. corrugata	0.00	0.015	1.259	1.123	+	"
P. fluorescens	0.00	0.007	1.165	0.806	+	"
P. nitroreducens	0.00	0.021	1.212	0.742	+	"
P. putida (2015)	0.00	0.010	1.006	0.971	None	Jeff Karn, USDA
P. putida paW1 with plasmid pWWO	0.00	0.015	1.424	0.738	+	R. Olsen, Univ. olf Michigan, MI
P. putida PPO200 paW1 cured of plasmid pWWO						R. Olsen, Univ. of Michigan, MI
Klebsiella pneumoniae	0.00	0.011	1.238	0.563	+	Isolated by Dr. Gunnison
Enterobacter gergoviae	0.00	0.009	1.03	0.918	+	"
Xanthomonas maltophila	0.00	0.020	1.035	1.097	+	"
Burkholderia sp. DNT	0.00	0.021	1.560	1.269	+	J. Spain, Tyndall Airforce Base, FL
Phanerochaete chrysosporium BKMF-1767	0.00	-	Dry wt (see Table 2)		None	C.A. Reddy, Michigan State Univ., MI

Control, no cell; killed cell, cells were boiled for 10 minutes before inoculation.

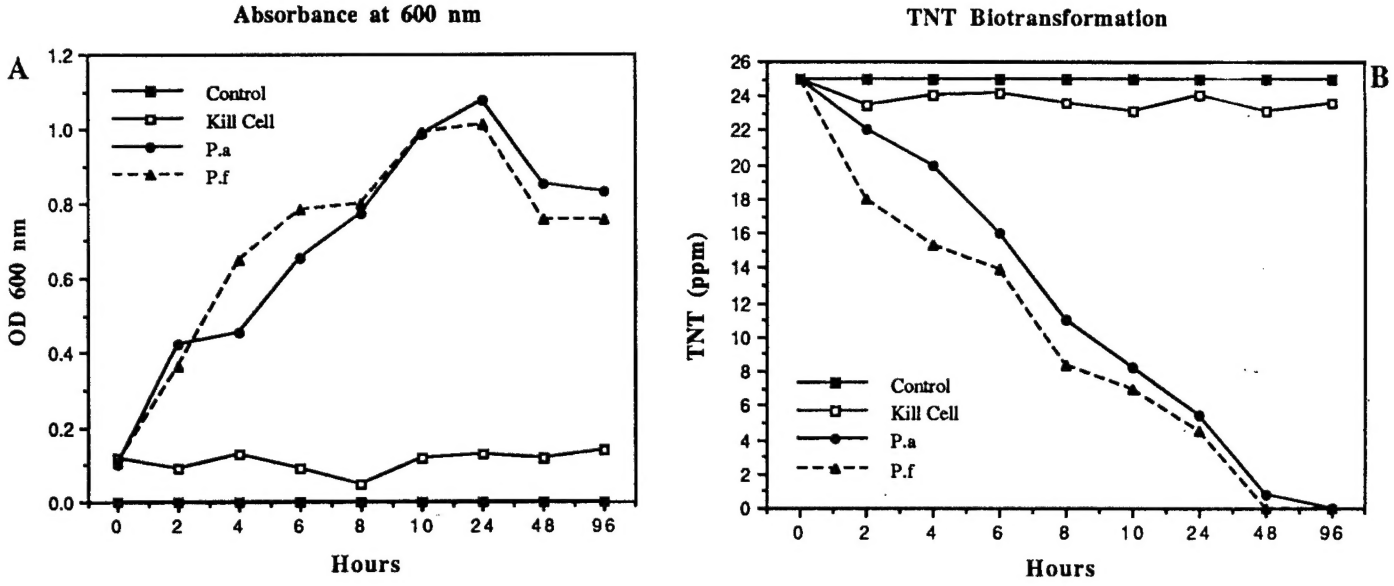

FIGURE 1: Growth patterns and biotransformation of TNT as function of time. *Pseudomonas aeruginosa* (P.a) and *P. fluorescens* (p.f) were grown in basal medium, 25 ppm TNT, and 0.5% NaoAc. (A) Shows increase in bacterial cell growth; (B) Shows decrease in TNT concentration. No change in cell growth or in TNT concentration are seen in control (no cell) or using killed cells (inactivate cell).

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TABLE 2: Growth Pattern of the Fungus *Phanerochaete chrysosporium* Under Different Media[a].

	Dry Weights of Mycelia (mg)		
Days of Growth	Without TNT[b]	With TNT[c]	% TNT Disappearance
1	13.7 ± 0.2	12.5 ± 0.4	34
2	14.6 + 0.3	13.2 + 0.3	81
3	14.9 + 0.3	13.8 + 0.4	95
7	16.2 + 0.4	14.9 + 0.6	100

a, Values are mean + s.d. for 3 replicate cultures.
b, Culture flask contained minimal salt medium without any carbon and nitrogen source. Supplemented with 22 mM glucose, 0.48 mM ammonium tartrate, 0.1 mM Tween-80, and 0.07 mM veratryl alcohol.
c, Culture flask contained minimal salt medium. In addition it contained 80% reduced amount 4.4 mM glucose, 0.09 mM ammounium tartrate, 0.02 mM Tween-80, and 25 ppm TNT.

Very minimal (less than 1 mg) growths were detected when cultures were without TNT but with the same amount of cometabolites. These data confirm that this fungal strain is utilizing TNT as the sole source of carbon and nitrogen.

TABLE 3: Composite data of assays for protein by different 5-days old bacterial cultures.

Names	Protein Assays			
	Without TNT		With TNT (25 ppm)	
	OD/595 nm	µg/ml	OD/595 nm	µg/ml protein
Enterobacter gergoviae	0.209	5.6	0.525	15.0
Pseudomonas aeruginosa	0.176	5.0	0.382	10.9
P fluorescens	0.266	7.6	0.432	12.3
P.nitroreducens	0.184	5.3	0.279	7.8
P. putida PaW1 with plasmid pWWO	0.321	9.1	0.552	14.8
P. putida PPO200 PaW1 cured plasmid pWWO				
Pseudomonas corrugata	0.170	4.9	0.180	5.1
Klebsiella pneumoniae	0.216	6.1	0.469	13.4
Xanthomonas maltophilia	0.136	3.9	0.655	18.7
Burkholderia sp. DNT	0.189	5.4	0.249	7.1
Pseudomonas 2015	0.084	2.4	0.094	2.7

Total proteins in supernatants of each cultures were estimated using Bradford method (Bradford, 1976). Similar studies done in 1 and 2 days old culture did not show significant difference in color developments with or without TNT in the growth medium. Protein was measured by the method of Bradford using BSA as a standard.

TABLE 4: Composite data of several assays for NO_2 release by different 5-days old bacterial cutlures._

Names	NO_2^- Release Assays					
	Without TNT			With TNT		
	Color (before assays)	OD/540 nm	µg/ml	Color (before assays)	OD/540 nm	µg/ml
Enterobacter gergoviae	Pale Brown	0.097	0.28	Bright yellow	0.186	0.53
Pseudomonas aeruginosa	Green	0.277	0.79	Light yellow	0.386	1.10
P.fluorescens	Light green	0.225	0.64	Light green	0.554	1.60
P.nitroreducens	Yellow	0.090	0.26	Bright yellow	0.362	1.00
P.corrugata	Green	0.173	0.49	Green	0.137	0.39
P.putida PaW1 with plasmid pWWO	White	0.023	0.066	Yellow	0.199	0.57
Klebsiella pneumoniae	Deep green	0.142	0.40	Deep green	0.297	0.76
Xanthomonas maltophilia	Yellow	0.026	0.074	Bright yellow	0.409	1.17
Burkholderia sp. DNT	White	0.019	0.054	White	0.271	0.77
Pseudomonas 2015	White	0.070	0.20	White	0.259	0.74

NO_2^- release assays were similarly tested in the supernatant only of the growth medium. Similar studies done in 1 and 2 days old culture did not show significant difference in color developments in with or without TNT in the growth medium however, on 5th day old bacterial culture media did show distinct color differences. NO_2^- release assay using KNO_2 as a standard was done as described (Joy and Hageman, 1966). Data shown here were with culture medium after cells were discarded by centrifugation.

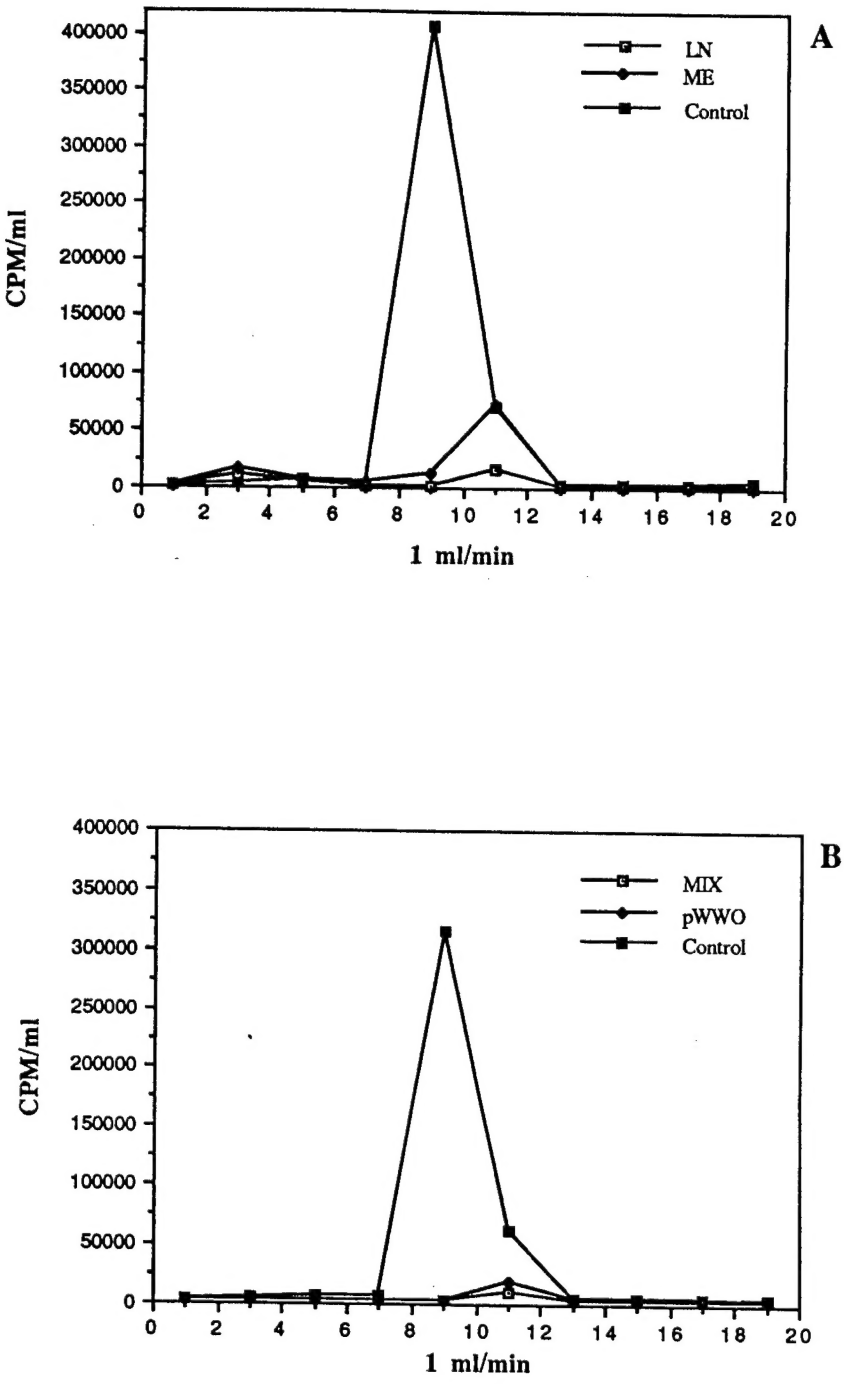

FIGURE 2: (A) HPLC elution profile of methylene chloride extract of [^{14}C]-TNT control, low-nitrogen, and malt extract culture of *P. chrysosporium* that was incubated with [^{14}C]-TNT (0.5 µCi) in 50 ml growth medium. (B) HPLC elution profile of a methylene chloride extract of samples (bacterial culture). 0.5 µCi (^{14}C) TNT in 25 ml growth medium. 2 ml fraction (1 ml/min) collected. Mixed cultures containing TOL gene (*P. putida* pWWO) + 2,4-DNT gene (*Burkholderia* sp.) was used. In both instances (bacteria and fungus) TNT disappeared and Am-DNT increased.

TABLE 5: ^{14}C-TNT Remaining in Two Growth Conditions of the Fungus *P. chrysosporium.*

		Counts per minute (cpm)	
	Day	Malt Extract	Low Nitrogen
Culture medium	0	725,400	704,700
Culture medium	13	308,320	352,700
Percentage		42.5%	50.0%

The actual ^{14}C-TNT cpm applied in the growth medium on the 3rd day after inoculation of spores which is regarded as "day 0" here. Similarly ^{14}C-TNT cpm were taken from the growth conditions after 13 days.

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TABLE 6: HPLC Analysis of TNT Under Two Growth Conditions Using *P. chrysosporium.*

	PPM of TNT Remaining	
Day	Malt Extract (25 ppm)	Low Nitrogen (ppm)
0	25	25
7	3.4	0
Biotransformation Percentage	86%	100%

Data shown above are actual concentration of TNT in ppm based on areas at seen in the HPLC (high performance liquid chromatography) data sheet at the same retention time 9.0 + 0.5 minutes using supplecosil™ LC18 column size 150x4.6 mm (Hewlett Packard HPLC System Model # HP1050). Mobile phase 55:46 (water:methanol). Flow rate 1 ml/min. Room temperature and wave length 254 nm.

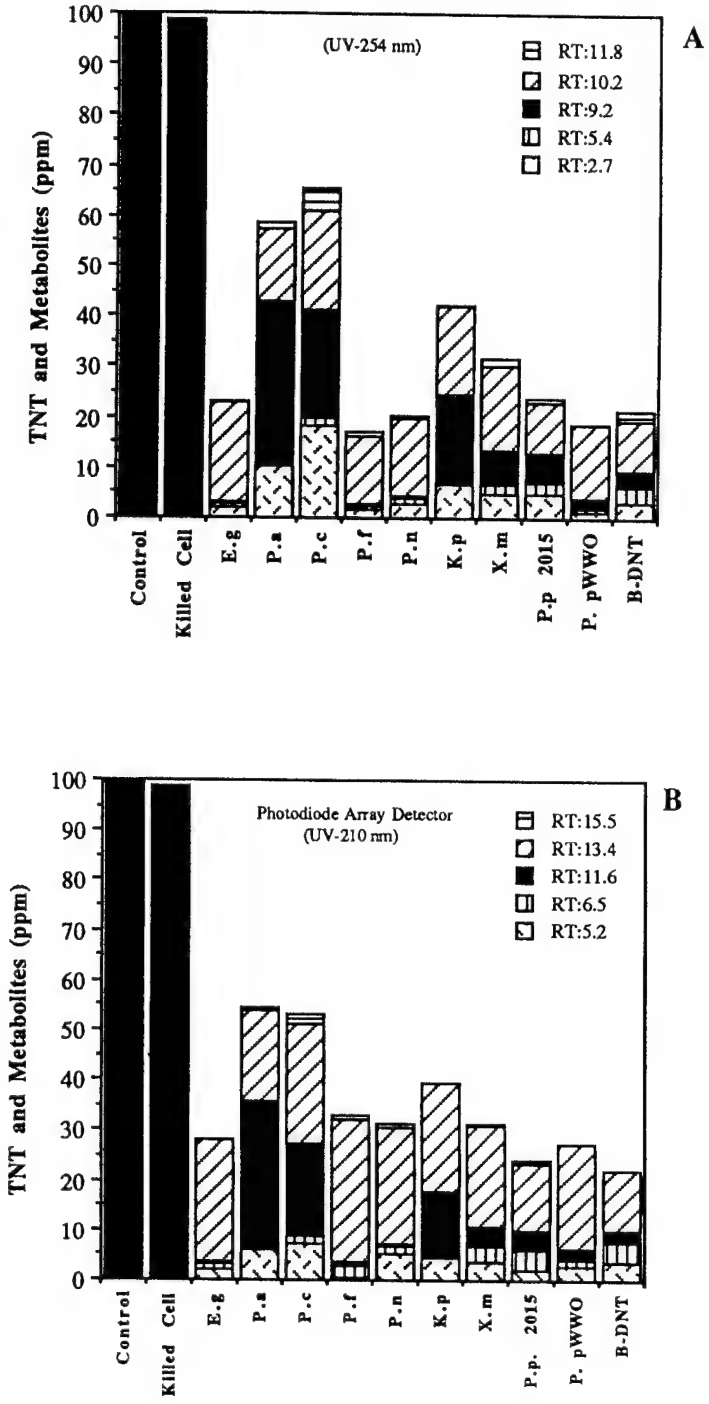

FIGURE 3: Composite histograms of TNT (100 ppm) biotransformation products. E.g., *Enterobacter gergoviae*; P.a., *Pseudomonas aeruginosa*; P.c., *P. corrugata*; P.f., *P. fluorescens*; P.n., *P. nitroreducens*; K.p., *Klebsiella pneumoniae*; X.m., *Xanthomonas maltophillia*; P.p 2015, *Pseudomonas putida* 2015; P. pWWO, *P. putida* pWWO; B-DNT, *Burkholderia* sp.DNA. RT (retention time) 11.8 in HPLC and 15.5 in photodiode assay array is identified as 2,4-DNT; RT 10.2 and 13.4 corresponds to Am-DNT; RT 9.2 and 11.6 correspond to TNT; other metabolites are yet to confirm.

^{14}C-TNT Mass-Balance Studies for Estimating Biodegradation

As described in the previous section, we have tried different approaches to study biotransformation of TNT using HPLC and GC/MS analysis. In this section we describe our attempts to study mineralization of TNT and its metabolites using ^{14}C-TNT. Mass balance studies of bacteria and fungal cultures indicates that $^{14}CO_2$ trapped in NaOH differed vastly. At our hand we have obtained about 22% of ^{14}C using the ^{14}C fungus *P. chrysosporium* whereas we found only 5% using mixed cultures of bacteria. $^{14}CO_2$ evolved in the fungus was vastly accelerated when O_2 was regularly flushed in the growth culture of the fungus. In case of bacterial culture flushing O_2 did not change any rate of $^{14}CO_2$ evolved.

Culture conditions: Bacteria cultures were incubated in basal salt medium, 0.5% sodium acetate and 0.5 µCi of ^{14}C-TNT (specific activity 21.58 mCi/m mol) and 25 ppm unlabelled TNT. Cultures were grown shaking at 37°C. Fungal culture (*P. chrysosporium*) was prepared by obtaining an aqueous suspension of spores of the fungus from three week old malt extract agar slants (1 ml/ 0.5 absorbance at 650 nm). Suspension of spores were inoculated in low-nitrogen medium and malt extract medium which were grown for six days. On day six of the fungal growth 0.5 µCi ($1.1x10^6$ dpm) of ^{14}C-TNT was added to the cultures. Low-nitrogen medium was used for ligninolytic condition in which peroxidases are produced and malt extract medium was used for non-ligninolytic cultures. Cultures were flushed with oxygen (99.9%) every other day for an incubation period of about 20 days. All controls contained culture medium minus bacterial and fungal inoculum. Culture medium (25 ml) was contained in a 250 ml CO_2 shaking flask (Gledhill, 1975). This flask system and set up was suggested by Dr. Gunninson.

TABLE 7: ^{14}C-TNT Mass-Balance Studies.

^{14}C-Counts	Control (%)	*P. putida* (pWWO) + Strain B-DNT (in percentage)	Fungus *P. chrysosporium*	
			By us	Literature*
$^{14}CO_2$ trapped in NaOH	0	4.05 ± 0.7	21.05	35.4
Water soluble metabolites	4.74 ± 1.5	49.28 ±3.9	52.91	25.1
Methylene chloride fraction	88.55 ± 2.7	15.01 ± 2.6	8.84	15.7
Bacterial cells	-	10.65 ± 2.2	-	-
Mycelia (fungus)	-	-	2.92	17.3
Wash flask	2.17 ± 2.8	10.26 ± 3.1	1.32	-
Total Radiolabels Recovered	**95.46%**	**89.25%**	**87.04%**	**93.5%**

Total ^{14}C-TNT added in culture media was 800,000 dpm.

*, Taken from Fernando et al., 1990.

Data shown in cells need to confirm using a biooxidizer, which will precisely determine amount of ^{14}C inside the cells.

VI. SEARCH FOR GENES CAPABLE TO BIOTRANSFORM AND/OR MINERALIZE TNT AND/OR IT'S METABOLITES

Different Approaches for Searching Genes:

We proposeto identify gene(s) capable of degrading TNT. TNT degradation seems to involve a number of genes. For example, four known genes are involved in degrading 2,4-DNT (Suen and Spain, 1993) and 9 more involved in toluene degradation (Franklin et al., 1981). We have obtained soil samples from Army (Dr. H. Tatem, Army, WES, Vicksburg, MS) which contain TNT, as high as 2000 ppm. This soil contain live bacterial cells in such highly contaminated TNT soil indicating that they are capable to use TNT for growth. We approached the problem by (i) making genomic libraries from both bacteria and from the fungus *P. Chrysosporium*. Genomic library of *P. chrysosporium* was a generous gift from Dr. Jacob Reiser (Institute of Biotechnology, Honggerberg, Zurich, Switzerland); and screening recombinant lambda plaques to express TNT degrading genes; (ii) acquiring a number of bacterial and fungal species which either alone or in combination can degrade TNT and Am-DNT, as tested by the most sensitive HPLC analysis; (iii) identifying homologs of some well characterized gene(s), 2,4-DNT using either DNA hybridization screening of the above libraries or by polymerase chain reaction (PCR) technology .

Genomic Libraries : We did not see hybridization between fungal RNA with a probe from a known TNT degradation gene. The fungal genomic library which we obtained from Dr. Reiser may have some TNT degrading gene(s) but the *E. coli* strain which served as a phage host did not grow in TNT media. However, we expect to obtain an *E. coli* strain which can grow in TNT containing media. This will be a good source of host to screen the genomic library again. If some good clones are obtained, we can compare their primary structure with those which we have identified recently by PCR. The shuttle vectors between *E. coli* and *Pseudomonas* were obtained from Dr. Schweizer, Alberta, Canada. The *Pseudomonas* host which we used for this purpose was pRS2015 which does not contain pWWO and thus does not give any background.

Genomic DNA library from Pseudomonas and other species: DNAs were isolated from *Pseudomonas* species. We have purified vector from *Pseudomonas putida* (pWWO) since expression of the TNT degrading genes will be optimum if the host is maintained as *Pseudomonas* sp. The DNAs of these species have been digested with restriction enzymes and insertsof 10-15 kb were partially ligated in pWWO vector. Results clearly showed that all strains (supplied by Dr. Gunnison) utilized TNT for growth. Several clones were developed while studying the expression in the shuttle vector followed by transformation to *P. putida* 2015 host. HPLC data showed only biotransformation of TNT to Am-DNT. We also did DNA-sequencing which confirmed using Blast database that they were not in the genes involved in degradation of nitroaromatic compounds.

Role of Mixed Cultures Containing Known Genes:

This approach has been most successful as shown in Figure 4. We have now a *Burkholderia* sp. Culture which can mineralize 2,4-DNT but not TNT. Similarly we have another culture *P. putida* (pWWO) which can mineralize toluene. These culture alone cannot degrade TNT but when mixed almost 80% TNT and Am-DNT disappeared.

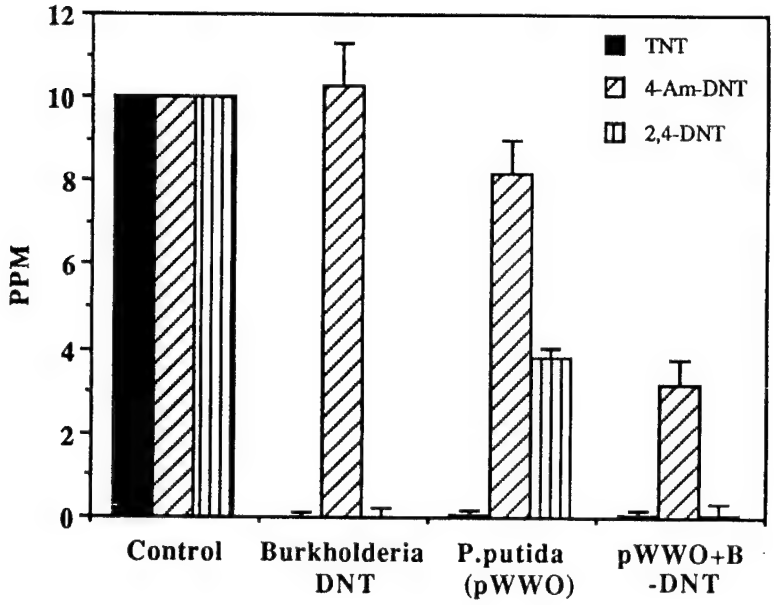

FIGURE 4: Biotransformation of mixed nitroaronmatic substances by single and mixed cultures containing 2,4-DNT gene cluster (*Burkholderia* sp.) and TOL gene cluster (*P. putida* pWWO).

Homology Studies of the 2,4-DNT Genes:

The plasmid DNAs of all 10 samples from various bacterial species showed homology to the DNT dioxygenase gene. Homology was also observed in the fungal genomic DNAs. Screening microbes for known 2,4-DNT gene probes and primers based on 2,4-DNT gene sequence such as dioxygenase, monooxygenase, oxygenase and nitroreductase enzyme systems which are in the levels of similarity between the *Pseudomonas* sp. The *dnt* A gene exhibited similarities to other dioxygenase enzymes especially those of the bacteria *Pseudomonas putida*. We have isolated the 6.8 kb size insert from the clone pJS6 (obtained from Jim Spain), was digested with *Eco*RI which revealed that pJS6 has one 14.7 kb, one 1.8 kb, one 0.6 kb and two 2.1 kb fragments. This was used as a probe (α-^{32}P labeled) and hybridized to ten different bacterial species containing plasmids, genomic DNAs and total RNAs. The fungal (*P. chrysosporium*) genomic DNAs and total RNAs were also hybridized with this probe.

PCR synthesis of a fraction of DNT dioxygenase gene showed that these showed that these sequences present in the *P. chrysosporium* fungal genome. We have sequenced the two PCR products and have compared these sequences in the GenBank. The primers were selected by use of a primer selection software. We tried nine different combinations of primers in order to detect any PCR products. Our results showed that the first set of primers MJ5 (5′-3′) and MJ4 (3′-5′) which contains 311 bp frag-ment and the second set of primers MJ6 (5′-3′) and MJ3 (3′-5′) which contains 220 bp fragment size successfully used on *P. chrysosporium* genome DNA as template. The results showed exactly same size using fungal genome but DNA sequences were different from *Pseudomonas* 2,4-DNT open reading frame 3 sequence. Thus indicating a totally different kind of gene. We are presently selecting other primers in other four open reading frames of DNT-A.

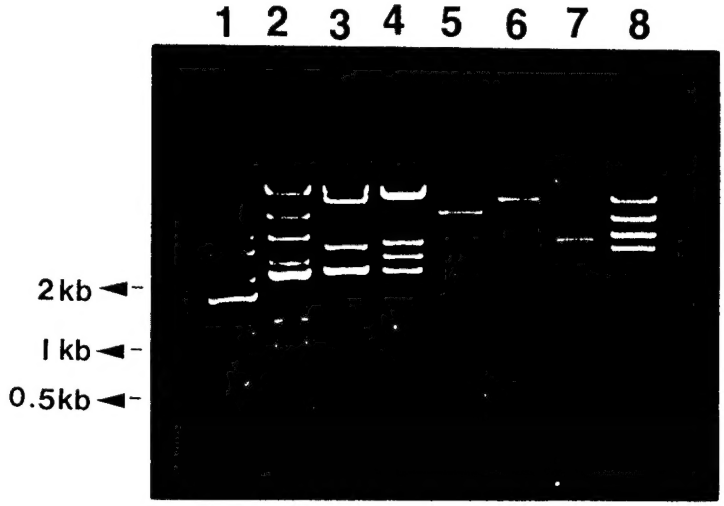

FIGURE 5: Isolation and purification of 2,4-DNT gene cluster probes for rapid screening of the presence of these genes in several other bacterial and fungal cultures.
Lane 1, 1 kb STD; lane 2, pJS6 digestion with *Eco*RI + *Nsi*I; lane 3, pJS8 digestion with EcoRI + Xho I; lane 4, pJS19 digestion with Eco RI; lane 5, purified 2,4-DNT dioxygenase gene (6.8 kb); lane 6, purified monooxygenase (10 kb); lane 7, purified oxygenase (3.4 kb); lane 8, high mass of the marker.

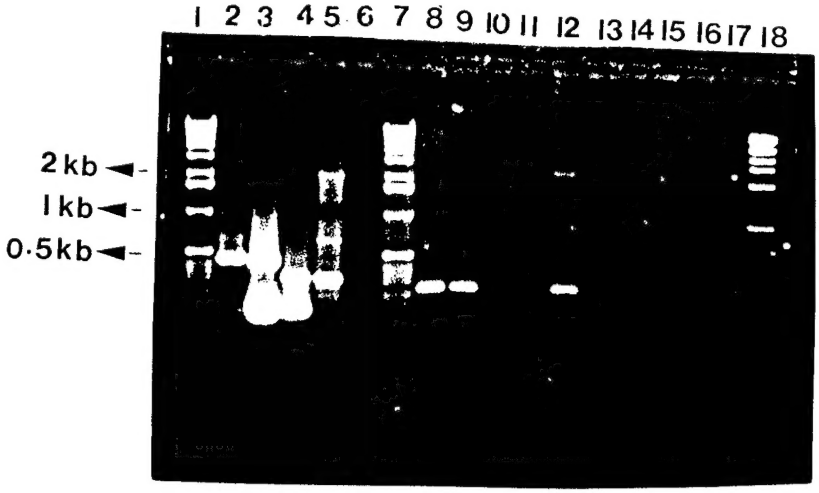

FIGURE 6: Screening for the presence of 2,4-DNT gene homology in the fungus *P. chrysosporium* and several bacterial cultures. Lane 1, 1 kb ladder; lanes 2-5PCR product using genomic DNA of fungus; lane 6, blank; lane 7, 1 kb ladder; lane 8, genomic DNAs of *E.gergoviae* PCR product; lane 9, *P. aeruginosa* PCR product from genomic DNAs; lane 10, PCR product of genomic DNAs of *K. pneumoniae* ; lane 11, PCR product of genomic DNAs of *Xanthomonas maltophilia* ; lane 12, PCR product of genomic DNAs of *P. putida* (pWWO); lane 13, *E. gergoviae* of plasmid PCR product; lane 14, *P. aeruginosa* of plasmid PCR product; lane 15, *K. pneumoniae* of plasmid PCR product; lane 16, *X. maltophilia* of plasmid PCR product; lane 18, high mass DNA marker.

Search for Am-DNT Genes from Highly Contaminated Soil With the Explosive TNT:

As mentioned before we have procured and screened several genes involved in the degradation Am-DNT. We have tried to procure two bacterial cultures that degrade Am-DNT as per recent publications (Alvarez et al., 1995; Duque et al., 1993). It is possible that those cultures may be available in future after authors have thoroughly studied them. We have therefore initiated an attempt to isolate Am-DNT degrading culture from a highly (2000 ppm) TNT contaminated soil given to us by U.S. Army (Dr. Tatem). In a preliminary attempt we have had isolated numerous colony forming units (CFU) from soil extract when spread on agar plates containing 50 ppm 2-Am, 4-DNT. It appears that several potential CFU are oxygen sensitive. We are therefore in the process of creating facilities for anaerobic microbiology. We intend to continue this search for Am-DNT gene(s) cluster in both aerobic and anaerobic microbes.

VII. ANCILLARY STUDIES: GENOTOXICITY STUDIES OF TNT METABOLITES.

TNT contaminated soil and underground water imposes serious and widespread environmental pollution. Toxicity of TNT is well established but not much known about TNT metabolites. It was natural therefore for us to ask question about the nature and severity of genotoxicity of TNT metabolites. Using latest state of the art PCR technology we have tested initially three metabolites of TNT such as 2-AM, 4-DNT; 2,4-DNT; and 3-NT by p53 gene mutation. p53 gene mutation has been correlated with carcinogenicity of toxic compounds by several researchers in recent times. We determined the toxicity of TNT metabolies to a hybrid Chinese hamster mouse neuroblastoma NG108 cell line (Dr. M. Nirenberg, NIH, Bethesda, MD). Studies involved: 1) studying cytotoxic effects (using cytotoxic 96™ kit, Promega) of TNT and its metabolites on NG108 cells and determing the lethal dose (or IC_{50}) and optimum exposure time; 2) isolation of RNA and RT-PCR (reverse transcripts polymerase chain reaction) of p53 "mutational hot spots" located in exon 5-9 using amplimers for this region for both control and exposed NG108 cells; 3) DNA sequencing to detect point mutation; 4) isolation of genomic DNA and amplification of p53 intron 5 using primers specific for detection of dinucleotide repeats and loss of heterozysity (LOH) as described by Jones and Nakamura (1992). Results showed that TNT and its nitroaromatic compounds such as Am-DNT, 4-NT were cytotoxic, among which 4-amino-DNT was the most toxic compound. The IC_{50} (concentration at which 50% cells die) was at a dose of 100 ppm when exposed for 7 hours. RT-PCR resulted in a 371 bp fragment amplification which is being sequenced for mutation detection. A study for LOH of the p53 DNA using specific primers yielded fragments of 114 bp indicating LOH due to mutation.

Exposing cell to 100 ppm of Am-DNT for 7 hours caused apoptosis as determined by annexin V-FITC and progidium iodide straining of cells as observed under fluoroscence microscopy. PCR-based assay of mutational hot spot of p53 tumor suppressor gene derived from cells exposed to 100 ppm of Am-DNT showed point mutation.

VIII. SUMMARY OF ACHIEVEMENTS

Several achievements are made through this initial collaborative project. These are: 1) creation of enormous capability at this HBCU in enhancing this research; 2) extensive studies on identification of TNT biotransformation products in several bacterial species and one fungal species; 3) identification of several genes which are capable of catalyzing degradation of metabolites of TNT in several microbial species, particularly in the fungus *P. chrysosporium*; 4) isolation and DNA sequence of these genes for probe development; and 5) PCR amplification of 2,4-DNT gene using *P. chrysosporium* genomic DNA as template; 6) DNA:DNA hybridization ^{32}P labeled 2,4-DNT dioxygenase gene (used as probe) to anneal with *P. chrysosporium* genomic DNAs; and 7) selection of proper combination of mixed genotypes of bacteria for very efficient degradation of TNT.

IX. REFERENCES

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X. PUBLICATIONS

Presentations from this Project (Abstracts Published)

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2. Dutta, S.K. James Stewart and Li-hua Hou. Dept. of Biol., Howard Univ., Wash. D.C. Microbial biotransformation of 2,4,6-trinitrotoluene (TNT) using *Pseudomonas fluorescens*. 1995 NIGMS Minority Programs Research Symposium, Hyatt Regency Washington Capitol Hill, Wash. D.C., October 11-15., 1995.
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9. H.N. Banerjee, M. Verma and S.K. Dutta. Dept. of Biol., Howard Univ., Wash. D.C. Alteration of p53 tumor suppresser gene expression by nitroaromatic organic compounds. 96th National Minority Research Symposium, Miami, FL, November 13-17, 1996.

XI. PAPERS IN PREPARATION AS OF 1996.

1. Dutta, S.K., L-H. Hou, Verma, M. and D. Gunnison. TNT biotransformation in several bacteria species: Search for genes.
2. Jackson, M.M., Banerjee, H., Sridhar, R. and Dutta, S.K. Biotransformation of nitroaromatic compounds by the white rot fungus *Phanerochaete chrysosporium* under non-ligninolytic condition.
3. Banerjee, H., Verma, M. and Dutta, S.K. Detection of genotoxicity of 2,4-Am-DNT by identification of dinucleotide repeat polymorphism at the Tp53 locus of MCF7 cells by PCR based assays.
4. Banerjee, H., Verma, M. and Dutta, S.K. Exposure of mammalian cells to 100 ppm of Am-DNT causes cytotoxicity and apoptotic changes.